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Synthesis of Ribosyl-ribosyl-adenosine-5',5'',5'''(triphosphate)—the Naturally Occurring Branched fragment of Poly ADP Ribose

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Poly-adenosine diphosphate ribose (PAR) is a branched biopolymer that occurs as a post-translational modification of proteins. In 1981 Miwa et al. determined the structure of enzymatically prepared branched PAR. We present the first synthesis of the same branched PAR fragment and show by NMR that the structure proposed by Miwa is correct.

Poly ADP ribosylation (PARylation) is an important post-translational modification in which negatively charged ADP-ribose units are transferred to an acceptor protein using NAD^+ (nicotinamide adenine dinucleotide) as a donor and PARPs (poly ADP ribose polymerases) as the involved enzymes[1, 2] (Figure 1). PARylation and the resulting polymers (PARs) are involved in many biological events such as DNA repair, transcriptional regulation and apoptosis[3]. PAR chains can be either linear or branched[4]. Linear PAR can grow to over 200 units in size, with a branching site occurring on average once every 20 to 50 elongation reactions[1]. While the knowledge on linear PAR is steadily growing, less progress is made with the role of branched PAR and its function is still unclear. There are a few reports on branched PAR after its discovery by Miwa et al[4] at the end of 1970s. For example, the branched and not the linear PAR chains bind most preferably to histones[5] and other nuclear proteins[6]. The branching point is reported not to be the endoglycosidic cleavage site of poly-ADP-ribose glycohydrolase (PARG)[7] which indicates there might be undiscovered enzymes that specifically recognize the branched PAR structure[8, 9]. In 1981 the chemical structure of the branching point of PAR was established as *O*-α-D-ribofuranosyl-(1'''→2'')-*O*-α-D-ribofuranosyl-(1''→2')-adenosine-5',5'',5'''-tris(phosphate) (Figure 1**, 1**) by Miwa et al[2]. They performed an enzymatic synthesis using NAD^+ and calf thymus nuclei, to get PAR *in vitro*. Subsequent hydrolysis of all the pyrophosphate linkages in PAR by treatment with snake venom phosphodiesterase led to the isolation of branched PAR fragment **1**. The configuration of **1** was determined by derivatization and with the aid of physicochemical techniques including gas chromatography, mass spectrometry, and ^{1}H-NMR spectroscopy[2]. Shortly after the structure elucidation, two different groups[10, 11] reported the existence of branched PAR *in vivo,* indicating that the branched PAR fragment made from enzymatic synthesis is indeed the naturally occurring product. Furthermore, enzymatic synthesis is widely applied to simulate *in vivo* conditions and to produce PAR[12-14]. In this respect, the organic synthesis of *O*-α-D-ribofuranosyl-(1'''→2'')-*O*-α-D-ribofuranosyl-(1''→2')-adenosine-5',5'',5'''-tris(phosphate) **1** is a challenging and valuable goal that can confirm this structure elucidation and will support future biological studies. As part of a program to develop synthetic methodologies towards PAR related molecules such as ribosylated amino acids[15], mono-ADP ribosylated peptides[16, 17] and ADP ribose dimer and trimer[18], we here present the synthesis and structural analysis of compound **1**. The synthetic route toward the *O*-α-D-ribofuranosyl-(1'''→2'')-*O*-α-D-ribofuranosyl-(1''→2')-adenosine-5',5'',5'''-tris(phosphate) **1** is guided by our earlier reported synthesis of the core motif of branched PAR[19] by adaptation of the protective group strategy and simultaneous introduction of three phosphate triester functions on the 5',5'', 5'''-primary hydroxyls of a suitably protected branched trisaccharide with phosphoramidite chemistry.

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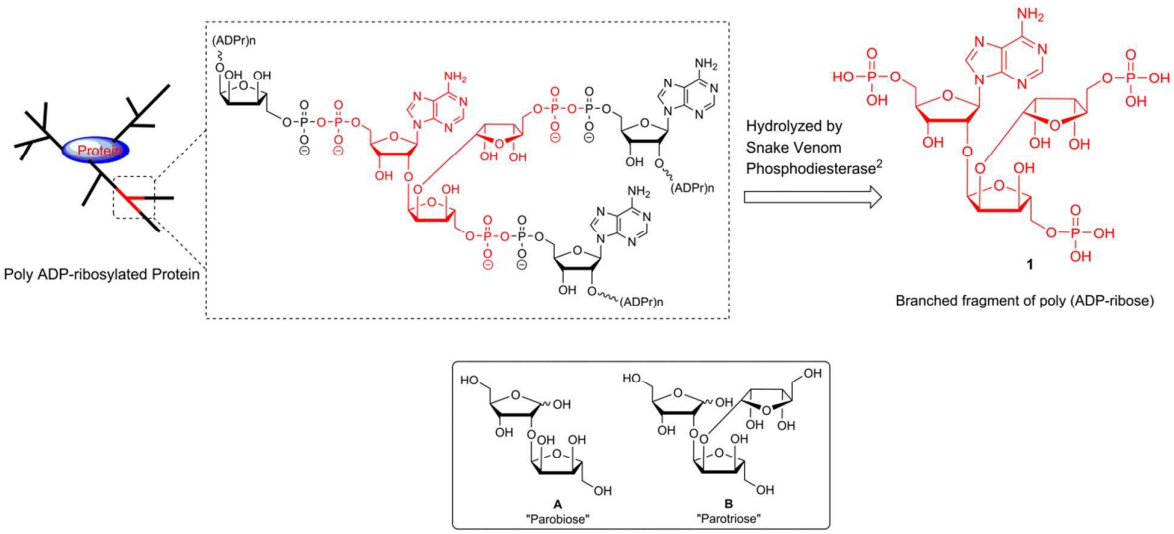
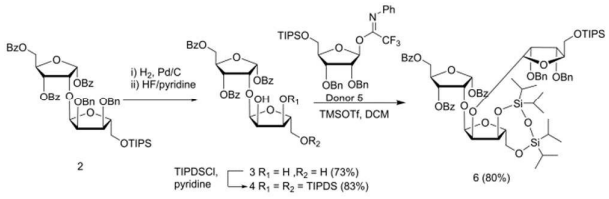


Figure 1. Structure of branched PAR fragment **1**. We propose to call disaccharide **A** "parobiose" and trisaccharide **B** "parotriose".

The first stage of the synthetic route comprises the introduction of two challenging 1,2 cis-α-glycosidic linkages by the synthesis of key intermediate **6**[20] (Scheme 1). We suggest to call the parent trisaccharide parotriose (PAR + triose, Figure 1, **B**) and the corresponding ribose disaccharide parobiose (**A**). The hydrogenolysis of the benzyl groups in fully protected parobiose, **2** (Scheme 1) obtained according to our earlier reported method[18] was followed by removal of (triisopropylsilyl) TIPS group with HF·Pyridine to give 2'',3'',5''-OH parobiose derivative **3** in good yield. It is of interest that the presence of the TIPS instead of the benzyl group at the 5'-OH of compound **2,** avoided glycosidic bond cleavage during hydrogenolysis as reported in our previous study[19], resulting in a significant improved yield and making a large scale synthesis possible. The 3'',5''-OH functions in triol **3** were selectively masked with the diol protecting TIPDS group by treatment with 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane ($TIPDSCl_2$) in pyridine to get alcohol **4**. Coupling of partially protected parobiose **4** with *N*-phenyl trifluoroacetimidate donor **5** afforded the fully protected parotriose **6** with complete α-selectivity and improved yield[19]. Subsequent introduction of adenine base requires a number of protective group manipulations. The benzyl-protecting groups in **6** should preferably be replaced because the adenine moiety would complicate hydrogenolysis[20]. However, we observed that the removal of benzyl ethers in **6** by Pd/C-catalyzed hydrogenation was tedious (more than 4 days) and was accompanied by ring opening or total cleavage of the TIPDS group. This side reaction could not be prevented by the use of other catalysts such as $Pd(OH)_2$. We presumed that the lability of the TIPDS group on the trisaccharides made the hydrogenolysis problematic but selective removal of the TIPDS in **6** could not be attained**.** Therefore, we treated **6** with an excess of $Et_3N{\cdot}HF$ for 24 h to remove all silyl groups. Hydrogenolysis of the thus obtained compound **7** using Pd/C in methanol for 24 h afforded compound **8**, provided with five hydroxyl functions, in high yield (Scheme 2). Readjusting the protection by installation of TBDPS groups on the primary hydroxyls of compound **8** and acetylation of the remaining secondary hydroxyls set the stage for introduction of N^6-benzolyadenine on the reducing end of parotriose **9**. Vorbrüggen type glycosylation using $HClO_4$-SiO_2 as catalyst and persilylated N^6-benzolyadenine proceeded completely β selective and furnished **10** in high yield[19]. The selective glycosylation on the N-9 position and not on the *N*-3 or *N*-7 positions was ascertained by UV-spectroscopy. Before three identical phosphate triesters could be installed on the 5',5'', 5'''-primary hydroxyls, protective group manipulation was required to ensure regioselective phosphorylation. Thus, saponification of the acetyl and benzoyl esters with aqueous NaOH in pyridine/ethanol gave intermediate **11**, allowing protection of the remaining free 5'-OH group with a TBDPS group. Surprisingly the reaction of the 5'-OH in **11** with TBDPSCl failed, but fortunately the equally suitable TBS group was introduced successfully using the more reactive TBSCl in pyridine. Subsequent acetylation of this intermediate gave fully protected **12**. After removal of the silyl groups by HF·pyridine all primary hydroxyl functions were released to give triol **13**, amenable to the simultaneous introduction of three di-tert-butylphosphate triesters. Treatment of **13** with 10 equivalents di-tert-butyl-*N*,*N*–diisopropylphosphoramidite using 1-methylimidazole and 1-methylimidazolium chloride as activator combination under strictly anhydrous conditions[21] and subsequent oxidation of the intermediate phosphite



Scheme 1. Synthesis of protected parotriose **6** from parobiose **2**

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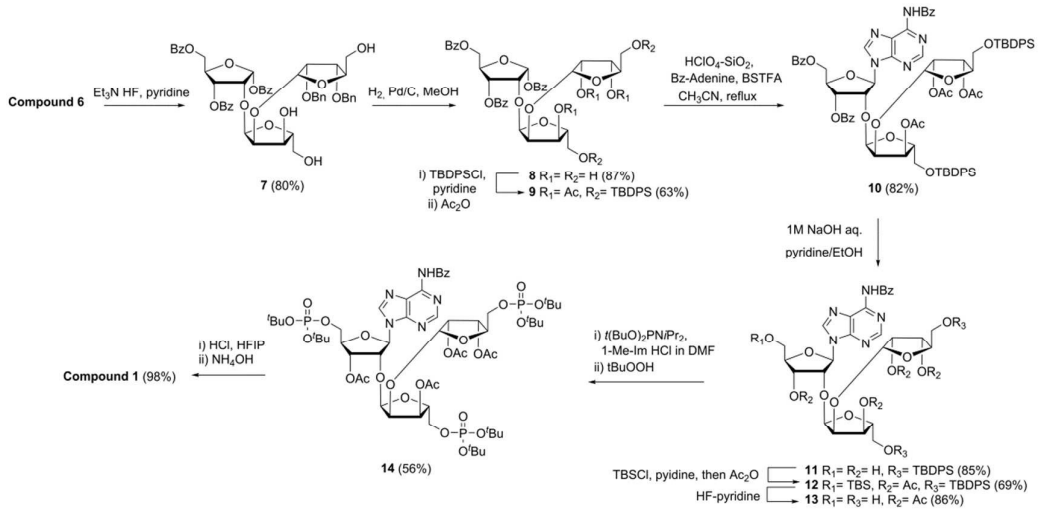

Scheme 2. Synthesis of branched fragment of poly ADPr **1**

triesters gave **14** in moderate yield. The low reactivity of 5'-OH of the adenosine moiety as noticed in the silylation of **11** decreases also the yield of the phosphitylation reaction as the formation of target **14** was accompanied by bis-phosphorylated product. In the final stage, the tert-butyl groups of the phosphate triester in fully protected **14** were removed with HCl/HFIP in 1 h followed by ammonolysis of the acyl groups to furnish tris-phosphorylated parotriose **1** in excellent yield.

Having target compound **1** at our disposal, we could compare the spectroscopic data of our synthetic product with those reported by Miwa[2] for the natural product (Figure 2A). In the first instance, we observed significant differences between the ^{1}H-NMR-spectra (see Supporting Information). We speculated that these differences originate mainly from a pH difference of the NMR samples, which in turn may be due to different isolation procedures. Our procedure involved global deprotection by ammonia treatment, followed by purification by HW-40 gel filtration using 0.15M NH_4OAc in H_2O as eluent under essentially neutral conditions. Contrary, Miwa firstly desalted the isolated product by DEAE-cellulose column chromatography, followed by column chromatography on phosphocellulose, which we could not easily attain. By doing this, Miwa obtained compound **1** as acidified sample (pH=3) while our product occurs in neutralized form (pH=7) as ammonium salt. To get a more accurate comparison, we changed the pH of our sample in D_2O from 7 to 3 by adding CD_3COOD. Under these conditions the NMR spectrum of our product and the one of Miwa proved to be virtually identical (Figure 2A vs Figure 2B). A small difference of multiplicity at R2''' and R3''' could be attributed to the slightly different field in our measurement. Overall the chemical shifts of all protons in our compound **1** are approximately 0.1 ppm upfield from those of Miwa's compound mainly because of they used DSS (sodium 2,2-dimethyl-2-silapentane-5-sulfonate) as reference while we did not. Taken together, we conclude that the synthetic compound and the isolated compound have the same chemical structure.

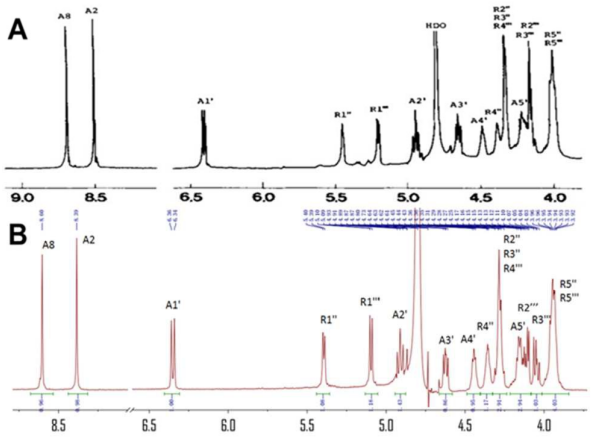

Figure 2. Comparison of 1H-NMR-spectra. (A) Isolated branched portion compound **1**, 270 MHz, in D2O (pH=3) as reported by Miwa et al. (B) Synthetic compound **1** from this work, 300 MHz, in CD_3COOD and D_2O (pH=3).

In conclusion, for the first time, O-α-D-ribofuranosyl-(1'''→2'')-O-α-D-ribofuranosyl-(1''→2')-adenosine-5',5'',5'''-tris(phosphate), a tris-phosphorylated branched PAR fragment was obtained by organic synthesis. Comparison of the ^{1}H-NMR spectra of this fragment and the naturally occurring product showed the same chemical shifts which means that the structure of **1** was identical to the naturally occurring compound[2] and that the regio- and stereochemistry of branching point of PAR was correctly elucidated by Miwa et al. Importantly, synthetic methodology in this work represents a next step to the future synthesis towards more complicated branched PAR fragments. Such fragments will be a valuable asset for future biological studies toward the biological function of branched ADPr. As last remark, we propose to give the disaccharide as occurs in linear PAR-chains parobiose and the native trisaccharide responsible for the branching points in PAR – parotriose, as trivial names.

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Total synthesis provided the first independent confirmation of the chemical structure of the branching point in poly-ADP-ribose.